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Generation and analysis of an RNA vaccine that protects against coxsackievirus B3 challenge

Isabelle P. Hunziker, Stephanie Harkins, Ralph Feuer, Christopher T. Cornell, J. Lindsay Whitton*

Department of Neuropharmacology, CVN-9, The Scripps Research Institute, La Jolla, CA 92037, United States

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Abstract

Coxsackievirus B3 (CVB3) is an important human pathogen that causes substantial morbidity and mortality but, to date, no vaccine is available. We have generated an RNA-based vaccine against CVB3 and have evaluated it in the murine model of infection. The vaccine was designed to allow production of the viral polyprotein, which should be cleaved to generate most of the viral proteins in their mature form; but infectious virus should not be produced. In vitro translation studies indicated that the mutant polyprotein was efficiently translated and was processed as expected. The mutant RNA was not amplified in transfected cells, and infectious particles were not produced. Furthermore, the candidate RNA vaccine appeared safe in vivo, causing no detectable pathology following injection. Finally, despite failing to induce detectable neutralizing antibodies, the candidate RNA vaccine conferred substantial protection against virus challenge, either with an attenuated recombinant CVB3, or with the highly pathogenic wt virus.

Keywords: RNA vaccine; Nucleic acid vaccine; Genetic immunization; Coxsackievirus; Antiviral vaccine; In vivo; Protective efficacy; Pancreatitis; Myocarditis; Picornavirus

Introduction

Coxsackieviruses belong to the *Picornaviridae* family and to the *Enterovirus* genus, which they share with polioviruses, echoviruses, and unclassified enteroviruses. They are classified, by their pathogenicity in newborn mice (Hyypia et al., 1993), into groups A and B which comprise 24 and 6 serotypes, respectively. The group B coxsackieviruses (CVBs) commonly infect humans, but the majority of infections (approximately 90%) are sub-clinical, or give rise to minor "flu-like" symptoms; however, these viruses can cause serious, and sometimes lethal, disease. CVBs are the most common infectious cause of human subacute, acute, and chronic myocarditis and dilated cardiomyopathy (Kishimoto and Hiraoka, 1994; Reyes and Lerner, 1985; Woodruff, 1980); they are a frequent cause of meningoencephalitis, particularly in neonatal life (Daley et al., 1998; Modlin, 1988); and they can cause severe pancreatitis (Arnesjo et al., 1976; Imrie et al., 1977; Lal et al., 1988; Parenti et al., 1996). Furthermore, although pancreatic infection often is limited to acinar tissue, CVB also has been implicated in type 1 diabetes mellitus (Clements et al., 1995; Ramsingh et al., 1997), which has a prevalence of approximately 0.2% in the USA (LaPorte et al., 1995). Despite the frequency of CVB infections, and the high associated morbidity and mortality, no vaccine against these viruses has yet been developed. In this study, we report our first attempts to develop an RNA-based vaccine against type 3 CVB (CVB3), using a murine model of CVB3 infection that faithfully recapitulates many of the aspects of human infection and disease. Both of the known receptors for CVB—the coxsackievirus adenovirus receptor [CAR;

* Corresponding author. Department of Neuropharmacology, CVN-9, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037. Fax: +1 858 784 7380.
E-mail address: lwhitton@scripps.edu (J.L. Whitton).

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(Bergelson et al., 1997, 1998; Tomko et al., 1997)] and decay accelerating factor [DAF (Bergelson et al., 1994, 1995; Shafren et al., 1995)]—are expressed in mice, and CVB3 infection of this specie induces pancreatitis (Mena et al., 2000; Ramsingh, 1997), meningitis (Gauntt et al., 1984; Sethi and Lipton, 1981), myocarditis (Fujioka and Kitaura, 2001; Kim et al., 2001), and myocardial scarring/ventricular dilatation (Gebhard et al., 1998).

Although antibodies are usually considered the predominant factor in controlling enteroviral infections (Kew et al., 1998; McKinney et al., 1987; Rudge et al., 1996; Yu et al., 2000), $CD8^+$ T cells also can play a part in limiting CVB titers (Henke et al., 1995, 2003; Horwitz et al., 2000). Therefore, a vaccine against CVB infection and disease should induce both antibodies and T cells and, in general, these two arms of the adaptive immune system are best stimulated by proteins that are abundantly synthesized within the host (Whitton and Oldstone, 2001). Other laboratories have designed vaccines against coxsackievirus-induced diseases. Some protection was conferred by several doses of an inactivated vaccine (See and Tilles, 1994), which appears unlikely to have induced strong T cell responses. Better protection was generated by vaccines in which protein synthesis occurred within the host cells, for example, live attenuated viruses (Fohlman et al., 1993; Gauntt et al., 1983) and DNA vaccines (Henke et al., 1998). However, these vaccines have some disadvantages. Live attenuated viruses expose vaccinees and their caregivers to the risk of revertant viruses which may cause the very disease they are intended to prevent; indeed, this is the major factor underlying the recent preference, in developed countries, for killed (injected, Salk) polio vaccine, over the live (oral, Sabin) vaccine. DNA vaccines contain strong eukaryotic transcriptional promoters, which if integrated into the host chromosome, may activate a cellular gene, with potentially deleterious consequences. Thus, we chose to evaluate RNA vaccines, which—unlike DNA—cannot be integrated into the genome; and which have been mutated to limit the production of infectious materials (thereby reducing the risk of reversion). RNA vaccines appear particularly appropriate for picornaviruses, which have positive-sense single-stranded RNA genomes; these molecules are infectious following in vivo injection, allowing us to readily construct a candidate vaccine.

The CVB3 genome is 7400 nucleotides (nt) in length (Genbank U57056: ref Knowlton et al., 1996), and consists of a 5′ untranslated region (5′UTR) of 742 nt, followed by one open reading frame of 6555 nt, that encodes the approximately 220 kDa viral polyprotein which is co- or post-translationally cleaved by virus-encoded proteases to generate intermediate products that can have significant half-lives, and may themselves have distinct functions. The polyprotein is considered to have three general regions, P1–P3. The P1 region, encoded near the N terminus of the polyprotein, contains the four viral capsid proteins (1A, 1B, 1C, and 1D, commonly known as VP4, VP2, VP3, and VP1, respectively); the P2 region encodes three non-structural proteins (2A–2C); and the P3 region yields four mature non-structural polypeptides (3A–3D). Two of the seven non-structural proteins are proteases (Kitamura et al., 1981; Krausslich and Wimmer, 1988). In the present study, we focused on the development of an RNA vaccine bearing point mutations at the cleavage site between proteins 2A and 2B. Transfection of this RNA into cells—in tissue culture or in vivo—was expected to produce CVB proteins and empty capsids, but no infectious virus. Using this candidate RNA vaccine, we addressed the following questions: (1) does the mutant genome retain the capacity to act as a template for viral polyprotein translation? (2) Do the mutations prevent the production of infectious virus following RNA transfections into tissue culture cells? (3) Is this candidate RNA vaccine safe, as determined by the outcome of in vivo RNA inoculation? (4) Can this RNA vaccine protect mice against challenge with recombinant CVB3 (rCVB3) or with wild-type CVB3 (wtCVB3)? (5) Are the protective benefits of vaccination mediated by CVB-specific neutralizing antibodies, as might be expected, given the natural history of the virus?

Results

Generation of a new plasmid pH3IH1 by site-specific PCR mutagenesis

Complete processing of the coxsackievirus polyprotein leads to the generation of 11 mature viral polypeptides. We wished to derive a coxsackievirus B3 (CVB3) RNA vaccine that: (i) would express a full-length mutant polyprotein, thereby ensuring that most of the virus proteome would be available for processing and presentation by the MHC class I pathway; (ii) would undergo sufficient autolytic processing to generate most of the mature virus proteins, including the capsid proteins, thus maximizing the chance that any antibodies induced would recognize the infectious virus; (iii) would have a reduced capacity to shut down host protein synthesis, increasing the chance that the transfected cell could present virus epitopes via its antigen presentation pathways; (iv) would not give rise to infectious particles, diminishing the risk to the vaccinee. To this end, we targeted the $3CD^{pro}$ cleavage site [consensus sequence, AXXQ˅G; (Andino et al., 1994; Hanecak et al., 1982; Mattion et al., 1994; Ypma-Wong et al., 1988)] at the junction between the coxsackievirus 2A and 2B proteins. Few studies of polyprotein processing have focused on coxsackieviruses, and our selection of this target site was based on findings with the closely related poliovirus. Mutations at the junction between the polioviral 2A and 2B proteins have been analyzed (Cohen et al., 1996), and the reported consequences appeared promising for our purposes: (i) lack of cleavage at the 2A/B junction has no deleterious effects

on cleavage at other sites in the polyprotein; (ii) despite the apparent normality of most processing, the mutant RNA does not generate infectious particles; (iii) the 2A protein has been implicated in the shut-off of host protein synthesis, for both enteroviruses (Krausslich et al., 1987) and other picornaviruses (Petersen et al., 1999), so preventing maturation of 2A might permit better maintenance of host cellular metabolism; (iv) mutation at the 2A/B junction results in markedly reduced RNA replication (Cohen et al., 1996) which may, in turn, diminish the risk that viral revertants will emerge. Therefore, a plasmid (pH3IH1) was constructed that contained point mutations at and near the 2A/B 3CD^{pro} cleavage site, using as a template the plasmid pH 3, which encodes a full-length infectious genome of CVB3 (Knowlton et al., 1996). The cloning strategy is outlined in diagrammatic form in Fig. 1. Mutations were introduced by PCR-based site-directed mutagenesis. Two pairs of PCR primers were synthesized: primers #1 and #3; and primers #4 and #2. Primer #1 contained a *Bsa*BI restriction site (GATNN^NNATC), unique in the pH3 plasmid, and had the sequence 5′CCCTAGGAGATACCA-ATCCCATGTGCTTTTAG3′ [corresponding to nucleotides (nt) 3565–3596 of the viral genome]. Primer #3 [5′CACTACCAGTTCCATTGCATCATCTTCCAGCCAC-GTTCCATTGCATCATCTTCCAGCCACAGGAGATCA-C3′] comprised complementary strand nucleotides 3751–3708, with two base changes (underlined). Primer #4 (nt 3708–3753; 5′GTGATCTCCTGTGGCTGGAAGATG-ATGCAATGGAACTGGTAGTGAA3′) is complementary to #3, and has complementary base changes (also underlined). Primer #2 (complementary strand nt 3850–3818; 5′TTGACCCACTAGTGATTCTTTCAGGAGGTTGAC3′) contained an *Spe*I restriction site (A^CTAGT), unique in pH3. For the initial PCR reactions, primer #1 was paired with primer #3, while primer #4 was paired with primer #2, and plasmid pH3 was used as template. The PCR products were then eluted separately, combined, and used as templates for the second-stage PCR, using primers #1 and #2. The resulting PCR product was digested with the enzymes *Bsa*BI and *Spe*I, then ligated into pH3 vector which had been cleaved with the same enzymes. The resulting plasmid pH3IH1 had the desired mutations [i.e., in position 3744 (A→T) which led to a replacement of glutamine by leucine, and in position 3747 (G→T) which replaced glycine with valine], and also a spontaneous mutation occurring in position 3749 (G→A; valine to methionine), which we accepted on the basis that it might further diminish proteolytic cleavage at the 2A/B junction. In addition, sequencing revealed a spontaneous mutation in the cloned fragment (T_{3709}→G; this mutation is silent at the amino acid level, and so is not included in the figure).

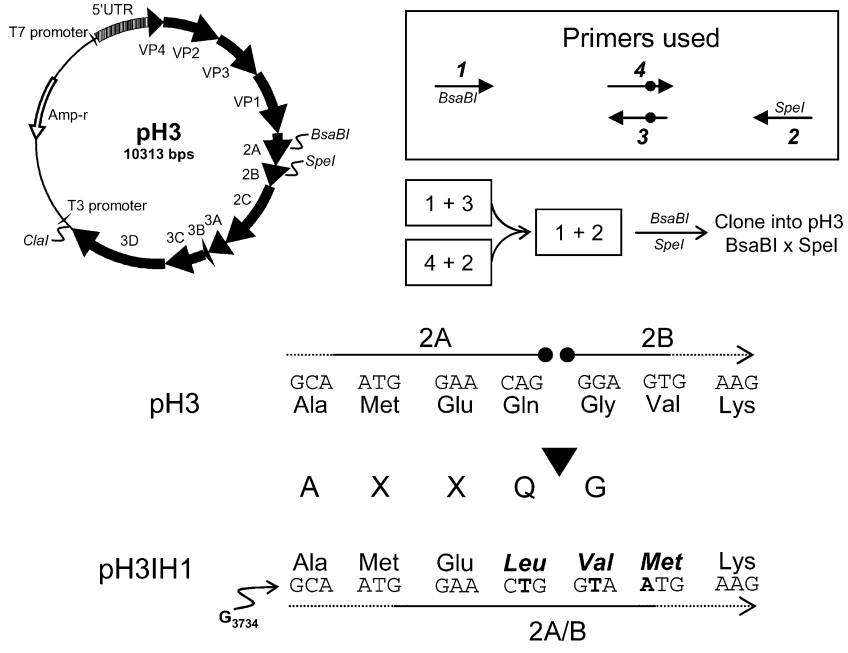

Fig. 1. Construction of plasmid pH3IH1, encoding CVB3 with a mutated cleavage site between the 2A and 2B proteins. The parental plasmid pH3 is shown, with each viral protein represented by an arrow. The 5′ untranslated region (5′UTR) is shown as a rectangle, preceded by a T7 RNA polymerase promoter, used to generate infectious RNA. Three unique restriction enzyme sites are included: *Cla*I (used to linearize the plasmid prior to in vitro RNA synthesis); and *Bsa*BI and *Spe*I, involved in the site-directed mutagenesis (see text). The four primers used for mutagenesis are indicated; the regions with altered bases are shown by black dots in primers #3 and #4, and the PCR cloning strategy is summarized. In the lower part of the figure, the nucleotide and amino acid sequences of pH3 that represent the wild-type 2A/2B junction are shown, along with the consensus cleavage site for the 3CD^{pro} protease, AXXQˇG. The equivalent region from the mutant plasmid pH3IHI is shown, with the three base changes in bold font, and the resulting three amino acid changes in bold italic font; the AXXQˇG motif is absent from this sequence.

In vitro translation of pH3IH1 RNA indicates that the mutant polyprotein is synthesized and that cleavage at the 2A/B site is interrupted

RNAs were transcribed in vitro using T7 RNA polymerase, and were added to an in vitro translation reaction. After a 4-h incubation, the labeled proteins were analyzed by gel electrophoresis and autoradiography, and the resulting data are shown in Fig. 2. The predicted processing patterns of the wt and mutant P2 proteins are shown in diagrammatic form. As indicated, the P2 protein normally is rapidly cleaved to generate 2A + 2BC; and the latter molecule subsequently is cleaved to generate 2B + 2C. All four of these bands are present in the pH3 lane (indicated by open dots). The mutant P2 protein, which lacks a cleavage site between 2A and 2B, should generate only two bands: a novel 2AB band, and the authentic 2C protein. Both are visible on the gel (solid dots). Furthermore, in the absence of a 2A/B cleavage site, one would predict that the 2BC, 2A, and 2B bands could not be generated; these bands are missing from the pH3IH1 lane. Taken together, these data indicate that pH3IH1 RNA is translated at high efficiency, and that processing of the resulting polyprotein occurs as expected.

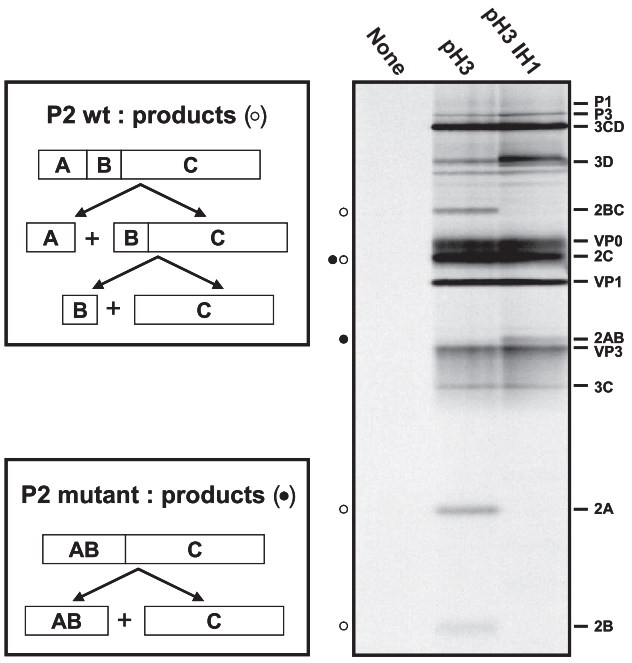

Fig. 2. In vitro translation of pH3 and pH3IH1 RNA. Cell free in vitro translation was carried out as described in Materials and methods, using as templates RNAs from pH3, or pH3IH1. As a negative control, no RNA was added to the reaction (left lane). The reaction mixtures were diluted in 2× lysis buffer, and 10 μl were run on a 12.5% polyacrylamide gel. The viral bands of interest are indicated to the right of the autoradiograph. Processing of normal P2, and predicted processing of mutant P2, are shown diagrammatically, and the expected products are indicated on the radiograph (wt, ○; mutant, ●).

pH3IH1 RNA fails to produce infectious progeny following transfection of HeLa cells

Next, we determined if the RNA could give rise to infectious virus. HeLa cells were transfected with 4 μg of either pH3IH1 RNA or pH3 RNA and, at 2, 4, 6, 8, 12, 18, 24, and 48 h post-transfection, supernatants were collected and analyzed for viral titers in standard plaque assays. HeLa cells were used in the RNA transfections because they express the viral receptor hCAR (Tomko et al., 1997); although this protein should not be required to permit successful RNA transfection, we wished to maximize the sensitivity of the assay by ensuring that any infectious virus produced following RNA transfection could infect susceptible cells, thus amplifying the sensitivity of the assay at later time points post-transfection. For the wild-type pH3 RNA, a low titer of virus was present in the supernate as early as 6 h after transfection and, by 18 h post-transfection, titers had risen to a plateau level of approximately 8×10^7 pfu/ml (Fig. 3A). In contrast, and despite the inbuilt amplification system which should permit the detection of low levels of virus, no infectious progeny could be identified, at any time point, in a standard plaque assay using supernates from HeLa cells transfected with pH3IH1 RNA. Furthermore, no virus was detected when these pH3IH1 supernates were incubated for a further 48 h with uninfected HeLa cells (not shown). Thus, mutations at the 2A/B junction appear to very effectively prevent the production of infectious CVB3, consistent with previous findings using a 2A/B cleavage mutant of poliovirus (Cohen et al., 1996).

Failure of 2A/B cleavage prevents RNA amplification in transfected HeLa cells

To identify the stage(s) at which infectious virus production might be blocked, we next determined if the RNAs could replicate following transfection of tissue culture cells. HeLa cells were transfected with 3 μg of either pH3IH1 RNA or pH3 RNA, and 0, 3, 6, 12, and 24 h later, RNA was harvested, immobilized on a slot–blot apparatus, and incubated with radio-labeled strand-specific RNA probes. The subsequent autoradiographs are shown in Fig. 3B; the intensities of the bands were determined using ImageJ software, and changes in genomic RNA content over time also are shown. The intensities of the bands representing genomic RNA (+ strand) in the pH3IH1 and pH3 tracks were essentially identical at t = 0, indicative of equivalent amounts of input RNA. However, the signal in pH3IH1-transfected cells decreased thereafter, and was barely detectable at 24 h post-transfection. In contrast, the genomic RNA signal in pH3-transfected cells dropped marginally in the first 3 h, then increased, at first gradually (6, 12 h) then dramatically (24 h). Anti-genomic (− strand) RNA also was readily detected 24 h after pH3 RNA transfection, but was not

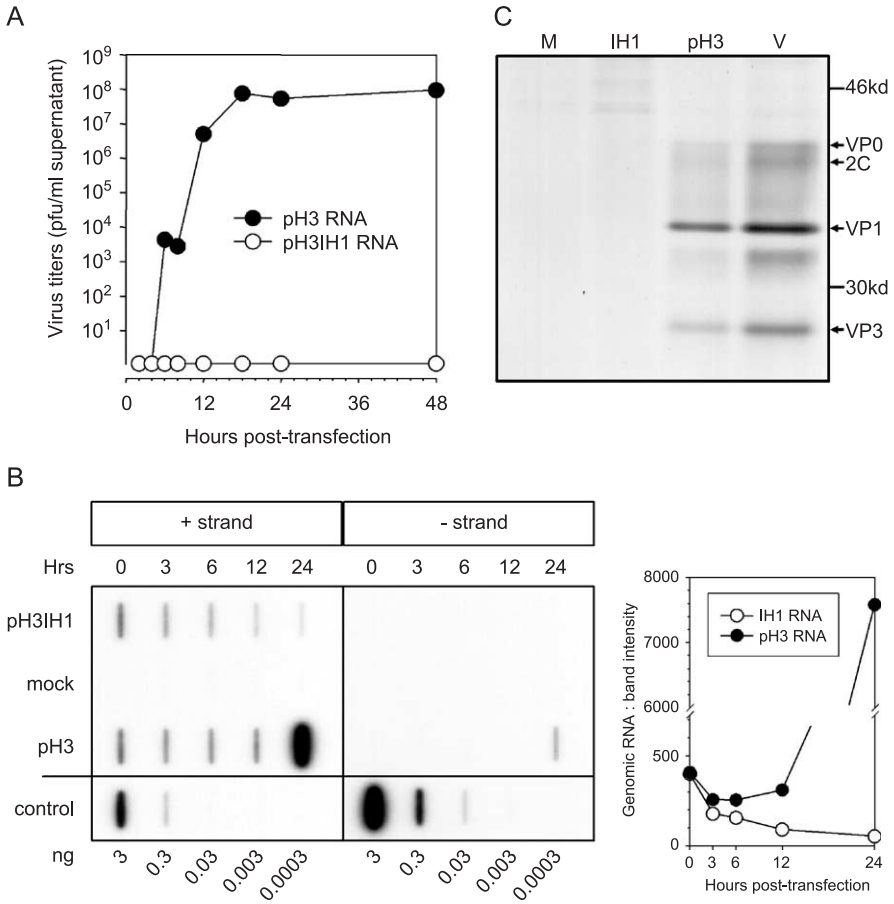

Fig. 3. Virus yield, RNA replication and protein synthesis following RNA transfection. Panel A: HeLa cells were transfected with either pH3 RNA or pH3IH1 RNA and, at the times indicated in the text, samples of supernatant were harvested and virus titers were determined. Panel B: RNA was harvested at various times after transfection, and the contents of CVB + and − strand RNA were determined by slot–blot analysis. The autoradiograph was scanned, and the band intensities were determined using ImageJ software; the values are plotted (*y* axis shows arbitrary units). Panel C: As described in Materials and methods, HeLa cells were infected with wtCVB3 or transfected with in vitro RNA transcripts from pH3 or pH3IH1, and a ^{35}S metabolic label was added. The viral products were immunoprecipitated with a polyvalent anti-CVB3 antibody, and were separated by electrophoresis through a 12.5% Tris-glycine polyacrylamide gel. The sizes of marker proteins are shown (Kd), and the viral bands are identified, to the right of the autoradiograph. M = mock-transfected; V = virus-infected.

detectable in pH3IH1-transfected cells. We conclude that the pH3IH1 RNA cannot be amplified following transfection into HeLa cells.

pH3IH1 RNA transfection fails to generate abundant virus protein

The production of viral proteins was evaluated by in vitro radio-labeling, followed by immunoprecipitation, as described in Materials and methods. The results are shown in Fig. 3C, for: mock-transfected cells (lane M); HeLa cells transfected with RNA derived from either the pH3 plasmid, encoding wtCVB3, or from the mutated pH3IH1 plasmid; and for virus-infected cells (lane V). Viral proteins were present in proteins harvested from infected cells, and from cells transfected with pH3, but proteins were not detected in cells transfected with the pH3IH1 RNA. Since the pH3IH1 RNA is an efficient template for protein synthesis (Fig. 2), the absence of protein in transfected cells is probably attributable to the lack of RNA amplification, leading to a dramatic difference in the quantity of template available for translation, compared to cells transfected with pH3 RNA.

pH3IH1 RNA is dramatically attenuated in vivo

From the viewpoint of vaccine safety, the demonstration that pH3IH1 RNA could not be amplified in tissue culture, nor produce virus, was encouraging. However, we felt it important also to determine whether this mutant genome was attenuated in vivo. As a positive control for infectivity of viral RNA, 13 C57BL/6 mice were injected with pH3 RNA. Two of these mice died within days of injection, and the others became ill, and were sacrificed on day 5, 7, or 9 post-injection. At each time point, the hearts and pancreata of these mice had high titers of virus (not shown), and most tissues showed marked pathological changes. A representative histological section of a pancreas harvested at 5 days post-injection is shown (Fig. 4, left panel), and reveals marked mononuclear cell infiltration, nuclear pyknosis, and

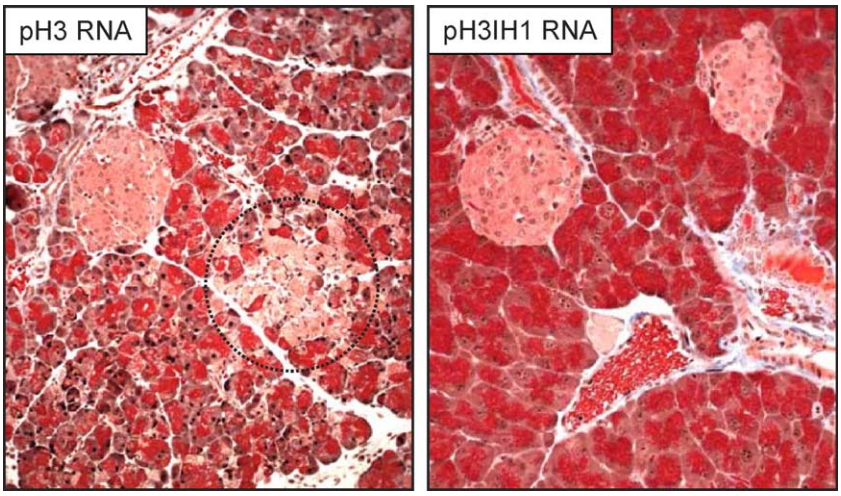

Fig. 4. RNA encoding wtCVB, but not the 2A/B mutant, results in pancreatitis. To determine the in vivo safety of the pH3IH1 candidate RNA vaccine, mice were inoculated with the indicated RNAs as described in Materials and methods, and tissues were harvested at various times post-injection (see text). The panels show trichrome-stained pancreatic sections (original magnifications 20×) obtained at 5 days post-injection. The dotted circle encloses an area of focal acinar cell destruction.

focal regions of acinar cell destruction (indicated by dotted circle); however, the islets of Langerhans were spared (as previously reported for wtCVB infection: Mena et al., 2000). The pathogenicity of pH3IH1 RNA was evaluated in a similar way, but using two strains of mice; normal C57BL/6 mice, and B cell knockout mice, which were selected because they are particularly susceptible to CVB3 infection (Mena et al., 1999). pH3IH1 RNA was inoculated into 10 mice of each strain, and the mice were sacrificed 7 days later. Virus was not detected in any of the tissues, and the hearts and pancreata were histologically normal (Fig. 4, right panel shows a representative pancreas). We conclude that the abrogation of cleavage at the 2A/B junction yields an RNA molecule which is non-pathogenic in vivo.

pH3IH1 RNA immunization confers significant protection against rCVB3 challenge

Our failure to detect virus protein in pH3IH1-transfected cells (Fig. 3) was disappointing, but the RNA is an effective template for translation in vitro, and it is known that only very small quantities of protein are required to induce strong immune responses. This, together with the demonstrated safety of the pH3IH1 construct, led us to evaluate the protective efficacy of the pH3IH1 RNA in vivo. Eight C57BL/6 mice were immunized with 15 μg of pH3IH1 RNA, three times at weekly intervals as described in Materials and methods. We have previously shown that nucleic acid vaccination can very rapidly induce protective immunity (Zhang et al., 2002) so, 1 week after the third immunization (4 weeks after the first), these mice, and a control group of 8 non-immune mice, were challenged with 10^6 pfu of eGFP-CVB3, a recombinant CVB3 encoding green fluorescent protein (Feuer et al., 2002). This virus is attenuated in vivo, and doses as high as 10^7 pfu are non-lethal in adult mice, even if delivered intracranially (Feuer et al., 2003). Three days later, the mice were euthanized, and viral titers in the pancreata were determined. As shown in Fig. 5A, high titers of virus (>10^8 pfu/g) were found in seven of eight non-immunized mice, and the remaining mouse had a titer of approximately 10^6 pfu/g. Four of the mice in the immunized group also had high titers; but two mice in this group had low titers (<10^5 pfu/g pancreas), and virus was undetectable in the pancreata of the remaining two individuals (indicated by asterisks). The difference between the immunized and non-immunized groups is highly statistically significant ($P < 0.0041$). Therefore, we conclude that the administration of pH3IH1 RNA can confer measurable protection against infection by an attenuated CVB3.

pH3IH1 immunization prolongs survival after wtCVB3 challenge

To test the protective efficacy of pH3IH1 RNA vaccination against a more stringent challenge, 10 C57BL/6 mice were immunized as described above, and were challenged with wtCVB3 (1.25×10^4 pfu, i.p.; >1 LD_{50}). A group of 10 non-immune mice was used as a control. Mice were observed daily for 1 month, and the survival data are shown in Fig. 5B. Fifty percent of immunized mice survived the challenge, compared with only 20% of naive mice; this difference is not statistically significant ($P = 0.17$). However, the vaccinees showed substantially prolonged survival; when the two groups are compared over the course of infection, the difference is highly significant ($P < 1.7 \times 10^{-5}$). All of the surviving mice displayed pathological changes in the pancreas, indicative of their having been productively infected with the wtCVB3.

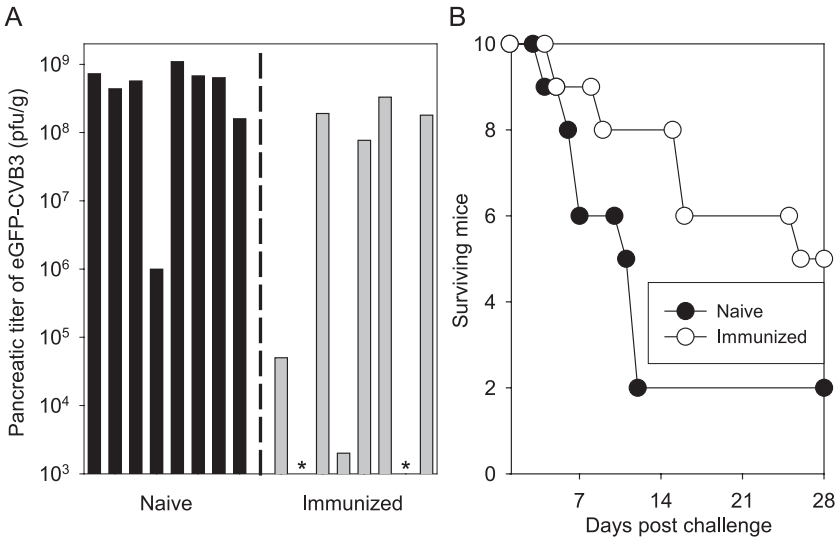

Fig. 5. RNA vaccine protects against both rCVB3 and wtCVB3 challenges. Groups of C57BL/6 mice were immunized with pH3IH1 RNA, and subsequently these groups, and control groups of age- and gender-matched non-immune C57BL/6 mice, were challenged with virus as described in Materials and methods. Panel A: Mice (8 per group) were challenged with 10^6 pfu of an attenuated rCVB3 and, 3 days later, were euthanized, and the viral titers in their pancreata were determined. Asterisks indicate samples in which no virus was present. The difference between the naïve and immunized groups is statistically significant (Student's t test, $P < 0.0041$). Panel B: Mice (10 per group) were challenged with 1.25×10^4 pfu (i.p.) of the highly pathogenic wtCVB3, and were observed daily for morbidity and mortality. The number of mice surviving over a 28-day period is shown. The difference in the survival curves is highly significant (Student's t test, $P < 1.7 \times 10^{-5}$).

Protection may not depend on neutralizing antiviral antibodies

Antibodies are known to play a key role in protecting against picornavirus-induced diseases, and individuals with defects in this arm of the immune system are at high risk of acute or persistent enteroviral infection (Davis et al., 1977; Geller and Condie, 1995; Hertel et al., 1989; Kew et al., 1998). Therefore, we wished to evaluate the possible role of neutralizing antibodies in the RNA vaccine-induced protective immunity shown in Fig. 5. One week after the third RNA immunization (3 weeks after the first), immediately prior to the viral challenges described above, blood samples were collected, and sera were evaluated for the presence of neutralizing antibodies, using a neutralization assay described in Materials and methods. Positive control sera, taken from a mouse that had recovered from wtCVB3 infection, contained a high level of neutralizing antibodies, but none of the sera from RNA-immunized mice showed any neutralizing activity (data not shown). Therefore, the origin of the protective immunity conferred by RNA immunization remains unclear, but may not depend on neutralizing antibody activity. The identification of CVB-specific $CD8^+$ T cells is difficult because the native viral epitopes are unknown. To circumvent this barrier, we previously constructed recombinant CVB3 encoding well-characterized epitopes from another virus, but we were unable to detect strong induction of epitope-specific $CD8^+$ T cells by this recombinant CVB3 (Slifka et al., 2001). Therefore, to attempt to evaluate virus-induced T cell activity, we measured a surrogate marker; serum levels of interferon-γ (IFNγ), an important antiviral cytokine that is produced by $CD8^+$ T cells. In all cases, IFNγ levels were below the level of detection in the sera of mice at 8 days after challenge with wt CVB3 (data not shown).

Discussion

Picornaviruses are important pathogens of humans (e.g., poliovirus, coxsackievirus) and ungulates (e.g., foot and mouth disease virus), and enteroviruses are thought to cause approximately 10–15 million symptomatic human infections annually in the USA (Sawyer, 2002). Most of these infections are self-limiting, but in some cases they cause substantial morbidity, and may even be lethal. Coxsackieviruses can cause fatal myocarditis, hepatitis, and meningo-encephalitis in adults (Bendig et al., 2001; Cree et al., 2003; Kamei et al., 1990; Longson et al., 1969; Marks et al., 1970), and are the leading infectious cause of myocarditis in the USA. These viruses also are highly pathogenic in neonates, who may succumb to myocarditis, pancreatitis, choriomeningitis, and encephalitis (Chalhub et al., 1977; Draganescu et al., 1980; Price et al., 1970). Moreover, associations have been drawn between CVB and sudden infant death syndrome (Cioc and Nuovo, 2002; Dettmeyer et al., 2002). Despite the high prevalence, and possible severe outcome, no vaccine is available to prevent CVB infection or disease. Several candidate vaccines have been designed, and tested in animal models. A polyvalent inactivated vaccine conferred protection, when administered in multiple doses (See and Tilles, 1994), and various live attenuated viruses [e.g., temperature-sensitive mutants (Gauntt et al., 1983), type-specific attenuated and chimeric

vaccines (Chapman et al., 2000; Fohlman et al., 1993; Hofling et al., 2000; Zhang et al., 1997)] also have conferred some degree of protection. As noted in the Introduction, live attenuated viruses pose a real risk of reversion. The attenuated viruses present in the Sabin vaccine rapidly evolve in the vaccine recipient (Dunn et al., 1990), and may infect caregivers or other contacts, causing the rare cases of poliomyelitis that occur in developed countries (Evans et al., 1985; Minor, 2003). Indeed, reversions of poliovirus vaccine strains have led to several outbreaks, some of which have been relatively localized (as in Haiti, in 2000: Kew et al., 2002), although others have reached epidemic proportions (as in Poland, in 1968: Martin et al., 2000). In part to avoid this risk, DNA vaccines have been constructed, and tested in animal models of coxsackieviral infection (Henke et al., 1998, 2001; Toniolo et al., 2002). These vaccines conferred some protection, but DNA vaccines carry with them their own (presently theoretical) risk: the possibility of harmful chromosomal integration. Depending on their design, RNA vaccines may avoid the risks alluded to above and, herein, we described the design, construction, and evaluation of an RNA vaccine against CVB3.

We designed a vaccine in which polyprotein cleavage at the 2A/B junction was interrupted. This strategy was chosen for several reasons. We considered the P1 region to be unsuitable for mutation because we wished all four mature capsid proteins to be produced, potentially permitting the induction of neutralizing antibodies; and we chose not to alter the P3 region, which encodes the major viral protease, as well as the viral replicase. We focused on the P2 region. The 2A protein plays a key role in regulating translational activity within the infected cells by redirecting the cellular translational machinery from cap-dependent translation [favoring host mRNAs] to IRES-dependent translation [favoring viral mRNA] (Ziegler et al., 1995). Therefore, limiting the activity of 2A (by preventing cleavage at the 2A/B junction) may maintain host cell translation, thereby facilitating the processing and presentation of viral antigens; however, this may be a double-edged sword, when viewed from a vaccine perspective, because there may be a corresponding reduction in IRES-driven translation, leading to a lower quantity of available viral antigen. The 2B protein also has been implicated in the shut-off of host protein translation (van Kuppeveld et al., 1996b), although most studies have indicated that—both for poliovirus and coxsackievirus—the 2B protein, and/or its 2BC precursor may be involved in viral replication (Cohen et al., 1996; Johnson and Sarnow, 1991; van Kuppeveld et al., 1996a, 1997).

The molecular details of the mutations within the candidate RNA vaccine are summarized in Fig. 1. Evaluation of translation products of the mutant RNA in a cell-free system indicated that the RNA acted as an efficient template for translation, and that the mutant polyprotein was processed exactly as predicted (Fig. 2); the 2A, 2B, and 2BC proteins—all of which rely on cleavage at the 2AB junction—are absent, and a new 2AB protein appears. Encouragingly, and in contrast to RNA representing the wt virus sequence, the candidate RNA vaccine did not yield infectious particles following transfection of susceptible HeLa cells (Fig. 3A); and more importantly, the in vivo inoculation of the pH3IH1 RNA failed to cause detectable pancreatitis (Fig. 4) or myocarditis (not shown), and no infectious virus was isolated from these tissues. This, again, was in stark contrast to the outcome of inoculating wtRNA, which almost invariably resulted in high viral titers, and concomitant pancreatitis (Fig. 4). This failure to produce infectious virus particles could have resulted from a defect at various stages of the virus life-cycle (e.g., replication, assembly, egress). We have shown that it is, most likely, caused by a failure of RNA amplification (Fig. 3B). Blockade at this very early stage of the life-cycle has both advantages and disadvantages. On the one hand, in the absence of RNA replication, there is a dramatically reduced probability that variant viruses will emerge; thus, the pH3IH1 vaccine is extremely safe. However, the reduced quantities of intracellular RNA also will diminish the available template for translation of the virus polyprotein, as shown in Fig. 3C. Despite this concern, the RNA vaccine described herein conferred significant protection against two viral challenges. When vaccinated mice were challenged with a high dose (10^6 pfu) of an attenuated virus, half of the mice appeared able to more efficiently suppress virus replication; however, the administration of the vaccine was not invariably beneficial because the remaining 50% of the mice showed virus titers similar to those observed in the control group (Fig. 5A). A more rigorous challenge, with >1 LD_{50} of wtCVB3, also provided strong evidence of protection (Fig. 5B): even though the eventual mortality was similar in both vaccinated and control groups, the former showed significantly prolonged survival ($P < 1.7 \times 10^{-5}$).

What might explain the RNA vaccine-induced immunity? Our understanding of the immune responses, which control CVB infections, is limited. Antibodies seem to play an important role in eradicating enteroviruses (Kew et al., 1998; McKinney et al., 1987; Rudge et al., 1996; Yu et al., 2000), and agammaglobulinemic humans are particularly susceptible to chronic infections with coxsackieviruses (Geller and Condie, 1995; Hertel et al., 1989; Palomba and Tovo, 1999). Furthermore, the inactivated (Salk) polio vaccine does not lead to protein synthesis within the cells of the vaccine recipient, and so this vaccine is unlikely to induce strong $CD8^+$ T cell responses; as a result, its protective efficacy has been attributed solely to the induction of virus-specific antibodies. Therefore, we anticipated that CVB3-specific neutralizing antibodies would underlie the protective effects of the RNA vaccine described in the present study, and we were surprised by our inability to detect any such antibodies in the sera of immunized mice after three RNA immunizations. Although there is no doubt that virus-specific neutralizing antibodies can protect against enterovirus infection, a possible role for the T cell arm of the

adaptive immune response should not be overlooked. High neutralizing antibody levels do not necessarily correlate with low virus titers (Woodruff, 1979), and antibodies were postulated to contribute little to the protection induced by a CVB DNA vaccine (Henke et al., 1998), raising the possibility that virus-specific $CD8^+$ memory T cells might play some part in vaccine-induced immunity against enteroviruses. Picornaviruses can induce $CD8^+$ T cell responses (Mandl et al., 1998) [although in general they appear to do so rather poorly (Slifka et al., 2001)], and $CD8^+$ T cells can play a part in limiting CVB titers (Henke et al., 1995). $CD8^+$ T cells may, in part, control CVB3 infections by their production of IFNγ, which diminishes CVB replication in tissue culture and in vivo (Henke et al., 2003; Horwitz et al., 2000); however, we were unable to detect this cytokine in the sera of CVB3-infected mice. Adoptive transfer studies, and analyses of transgenic knockout mice deficient in various aspects of the immune response, are currently in progress, and together should allow us to identify the immunological component(s) responsible for protection.

Materials and methods

Mice and tissue culture cells

Male C57BL/6 mice (H-2b MHC haplotype) and B cell knockout mice (Kitamura et al., 1991) were obtained from the Scripps Research Institute (TSRI) breeding facility, and were between 8 and 16 weeks of age when experiments were initiated. The HeLa cells used for virus titration and growth, and for RNA transfections, were a kind gift from Dr. Rainer Wessely (formerly at University of California, San Diego) and were maintained in Dulbecco's modified Eagle medium (DMEM; GIBCO BRL, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete DMEM).

Viruses and plasmids

The coxsackievirus B3 used is a plaque-purified isolate of the cardiopathogenic Woodruff strain of CVB3 (designated H3: Van Houten et al., 1991). An infectious clone of this virus is available (Knowlton et al., 1996), and the plasmid (pH3), generously provided by Dr. Kirk U. Knowlton (University of California, San Diego), was used as the parental plasmid for the construction of the candidate RNA vaccine pH3IH1. The recombinant CVB3 encoding enhanced green fluorescent protein (eGFP-CVB3), used as a challenge virus in the present studies, was constructed in this laboratory as previously described (Feuer et al., 2002). Coxsackieviruses (wild-type and recombinant) were propagated in HeLa cells (moi = 0.016). Sixteen hours post-infection, the culture medium was removed, and the monolayer of infected cells was frozen on dry ice. Thereafter, the cells were disrupted by three cycles of freezing and thawing. Cell debris was removed by centrifugation, and aliquots were stored at −70 °C. The titer of infectious virus particles was determined by a standard plaque assay on HeLa cell monolayers.

In vitro RNA transcription

The DNA template (pH3 or pH3IH1) was linearized with the restriction enzyme *Cla*I (New England Bio Labs, Beverly, MA), phenol-extracted, precipitated, and resuspended in sterile distilled water. One microgram of this DNA was used as a template for transcription, using the mMessage mMachine in vitro transcription kit (Ambion, Austin, TX). Transcription was carried out according to the manufacturer's directions, then the template DNA was removed by the addition of 1 μl of DNase, followed by incubation at 37 °C for 15 min. RNA was recovered by lithium chloride precipitation, as described in the instruction manual.

In vitro translation

In vitro translation reactions were carried out as described (Molla et al., 1991). Uncapped RNA was produced using the MAXIscript kit (Ambion). For the translation, 65% of S10 extract from HeLa cells, 1× "All four" buffer [10× buffer: 300 μl 1 M creatine phosphate, 300 μl 2 M KOAc, 100 μl creatine kinase (40 mg/ml in 10 mM HEPES–KOH, pH 7.4), 155 μl 1 M HEPES–KOH pH 7.4, 100 μl ATP, and 25 μl of each CTP, GTP, and UTP (each from 0.1 M stock from Amersham Biosciences, Piscataway, NJ)], 400 ng RNA, and 2 μl of ^{35}S-methionine (Amersham Biosciences) were mixed and used in 20 μl reactions. The translation was performed at 30 °C for 4 h, after which 2× lysis buffer was added, and the reactions were heated to 90 °C for 5 min. Ten microliters of the reactions were loaded and run on a 12.5% polyacrylamide gel. The gel was dehydrated with three washes using DMSO for 45 min each, and finally impregnated with 20% PPO in DMSO for 1 h. Another wash in running H_2O followed for 1 h, before the gel was dried on a gel-dryer at 65 °C overnight and finally exposed to a photographic film for 16 h.

Transfection of HeLa cells

To determine the infectious status of the RNA molecules, and to evaluate viral protein production in tissue culture, HeLa cells (at 70–80% confluency in a 6 well-plate) were transfected with 3–6 μg of the indicated RNA using Lipofectin and OPTI-MEM (both GIBCO BRL, Invitrogen) according to the manufacturer's directions; in our hands, 25–50% of HeLa cells express the desired gene products after transfection of nucleic acids. After 1 h, the transfection mixture was aspirated, and replaced by complete media; this

time point defined the beginning of the post-transfection period.

Evaluation of CVB3 RNA replication by slot–blot

HeLa cells were transfected with RNA as described above, and were harvested at 0, 3, 6, 12, and 24 h post-transfection. RNA was extracted using TRIzol LS Reagent (Invitrogen) according to the manufacturer's directions. RNA samples were dissolved in an aqueous solution containing 50% formamide (J.T. Baker Inc., Phillipsburg, NJ), 7% formaldehyde (Fisher Scientific, New Jersey, NJ), and 1× SSC and incubated at 68 °C for 15 min. Then, two volumes of 20× SSC (Ambion) were added, and each RNA sample was loaded onto a nitrocellulose sheet in the slot–blot manifold. After washing twice with 10× SSC, the nitrocellulose was removed, baked for 2 h at 80 °C in a vacuum oven, and crosslinked in a Stratalinker at 200 joules for 1 min. The nitrocellulose was pre-hybridized at 68 °C for 2 h in 50% formamide, 6× SSC, 5× Denhardt's reagent, 0.5% SDS (Ambion), and 100 μg/ml boiled, denatured salmon sperm DNA. Radiolabeled probes, specific for either the genome or antigenome, were added at 1×10^7 cpm/ml, and the blots were incubated at 68 °C for approximately 20 h, after which the nitrocellulose was washed three times: at RT using 1× SSC, 0.1% SDS; at 68 °C in 0.2× SSC, 0.1% SDS; and finally in 0.1× SSC, 0.1% SDS. The blot was exposed to Kodak film at −70 °C with an intensifying screen. The intensities of the bands on the resulting autoradiograph were quantified using the public domain software ImageJ (available at NIH web site http://rsb.info.nih.gov/ij/).

Evaluation of protein synthesis in transfected cells by ^{35}S metabolic labeling and immunoprecipitation

At 16 h post-transfection (or, as a positive control, at 16 h after infecting cells at a multiplicity of infection of 0.02), HeLa cells were washed with saline and starved for 30 min in methionine- and cysteine-free DMEM containing 10% FCS at 37 °C. 200 μl of ^{35}S-labeled amino acids (MP Biomedicals, Irvine, CA) were added (0.3 mCi/ml final activity), and a 1-h incubation at 37 °C followed. For subsequent immunoprecipitation, supernatants were aspirated and discarded, and the cells were lysed in 1 ml of RIPA lysis buffer (0.5% Nonidet P-40, 0.5 M EDTA, 5 M NaCl, 10% SDS, 1 M Tris [pH 8.0], 5 mM β-mercaptoethanol in H_2O). One microliter of a polyvalent anti-CVB3 antibody was added to 500 μl of each lysate, and the solutions were incubated, with gentle rotation, at room temperature for 2 h. Thirty microliters of protein A agarose beads (Sigma, St. Louis, MO) were added per 501 μl, and the samples were rotated for an additional hour. The beads were washed five times with the RIPA lysis buffer without β-mercaptoethanol, resuspended in 30 μl of 1× SDS buffer, frozen, then boiled for 5 min, and applied to a 12.5% polyacrylamide gel, as described in the text. The gel was dehydrated with three washes using DMSO for 45 min each, and finally impregnated with 20% PPO in DMSO for 1 h. Another wash in running H_2O followed for 1 h, before the gel was dried on a gel-dryer at 65 °C overnight and finally exposed to a photographic film.

In vivo RNA inoculation

RNA was suspended in sterile PBS at a concentration of 300 μg per ml, and 50 μl (15 μg) were injected into the tibialis anterior muscle. For studies of RNA infectivity, samples were harvested at the indicated times post-injection. For studies of RNA immunization, two boosts of RNA were administered as described above, at weekly intervals, and the mice were challenged with virus 1 week later.

CVB3 infection of mice (virus challenge)

Mice were infected by intraperitoneal (i.p.) injection of 0.5 ml serum-free medium containing the stated amounts of wtCVB3 or recombinant eGFP-CVB3, and were monitored daily for morbidity and mortality, or were euthanized at the indicated time points post-infection.

Organ preparation for titration and histology

The hearts and pancreata were removed from the mice immediately after euthanasia. Each organ was divided into two approximately equal portions, one of which was placed into a cryotube and fast-frozen in dry ice for later virus titration. The other portion of each organ was fixed in 10% normal buffered formalin, and processed for histological analyses. Five micron paraffin sections were prepared, and were stained either with hematoxylin and eosin (H&E) or with Masson's trichrome.

Plaque assays (viral titration)

HeLa cells were plated in six well plates ($5–7.5 \times 10^5$ cells/well) and incubated at 37 °C with 5% CO_2 for approximately 24 h, at which time they reached approximately 70–90% confluency. The weight of each frozen organ sample was determined, then the tissue was homogenized in 1 ml of DMEM, and 10-fold serial dilutions were prepared in DMEM. Media was aspirated from the 6-well plates, and 400 μl of each serial dilution was added to individual wells. Plates were incubated at 37 °C for 1 h, with gentle rocking every 15 min. Next, cells were overlaid with 4 ml of 1× DMEM in 0.6% agar (1:1 mixture of 2× DMEM at 37 °C and 1.2% agar at 55 °C). At approximately 48 h post-infection, cells were fixed in methanol/acetic acid (3:1 v/v), and agarose plugs were removed. The monolayers were stained with 0.5% crystal violet in 20% ethanol, rinsed in tap water, and plaques were counted. The titer (in pfu/g of

organ) was calculated based on the known weight of each tissue sample.

Virus neutralization assay, and measurement of serum IFNγ

Blood was collected from the tail and, after clotting, the serum was recovered. Sera were incubated at 56 °C for 30 min to inactivate complement, then were serially diluted in complete DMEM. Four hundred microliters of each dilution was combined with 100 μl of DMEM containing 100 pfu of CVB3. These materials were incubated, with gentle rocking, at 4 °C for 2 h. To determine if the viral particles had been neutralized by the mouse sera, a plaque assay was carried out as described above. Briefly, the samples were applied to HeLa cells at approximately 70–90% confluency, incubated for 1 h at 37 °C, then overlaid with 1× DMEM in 0.6% agar. Approximately 48 h later, cells were fixed and stained as indicated above. For each serum, the neutralizing antibody titer was defined as the dilution of antibody at which the number of plaques was reduced by 50%. Serum IFNγ was measured using the mouse IFNγ ELISA kit from eBioscience (San Diego, CA).

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